A Liquid Chromatography–Tandem Mass Spectrometry Method for the Determination of 5-Fluorouracil Degradation Rate by Intact Peripheral Blood Mononuclear Cells

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Abstract: 5-Fluorouracil (5-FU) is a major chemotherapy drug used for the treatment of tumors. It is catabolized mainly by dihydropyrimidine dehydrogenase, and patients with a complete or partial deficiency of dihydropyrimidine dehydrogenase activity are at risk of developing severe 5-FU–associated toxicity. The aim of this study was to demonstrate that intact peripheral blood mononuclear cells (PBMCs) can be an effective model to evaluate the degradation rate of 5-FU. We developed a sensitive and specific liquid chromatography–tandem mass spectrometry method to measure in vitro the rate of 5-FU degradation by intact PBMC. 5-FU degradation rate was determined by measuring the decrease of a fixed amount of 5-FU (10 μg/mL) added to a solution of PBMC, after 2 hours incubation, expressed as nanogram per milliliter of 5-FU degraded per minute × 10^6 cells. Freshly prepared intact PBMC can degrade efficiently in vitro–added 5-FU. The assay consists of 3 steps: (1) PBMC isolation from peripheral blood, (2) PBMC incubation with 5-FU in vitro for different times, and (3) determination of 5-FU amount to calculate the degradation rate. 5-FU was analyzed by a Q Trap 2000 triple quadrupole/ion trap mass spectrometer in the multiple-reaction–monitoring modes. The chromatographic separation was accomplished using a C_{18} column with a run time of 16 minutes. By analyzing samples from 39 patients with no 5-FU toxicity, the mean 5-FU degradation rate was 1.85 ± 0.50 ng·mL^{-1}·min^{-1} × 10^6 cells. The assessment of a test to measure 5-FU degradation rate in PBMC of patients before 5-FU administration could represent a prescreening method for evaluating the possible toxicity of this drug as an aid to set up a personalized medicine approach for each patient.

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5-Fluorouracil (5-FU), an analogue of uracil acting as a pyrimidine antagonist, is a major chemotherapy drug used for the treatment of gastrointestinal, breast, ovary, and head and neck cancers, with reasonable results.[1] The metabolites of this drug interfere with normal pyrimidine metabolism through 3 main mechanisms of action: (1) fluorodeoxyuridine monophosphate competes with uracil in binding thymidylate synthase and folate cofactor. This decreases thymidine production and thus inhibits DNA synthesis and repair and ultimately decreases cell proliferation. (2) Fluorodeoxyuridine triphosphate is incorporated into DNA, thus interfering with DNA replication. (3) And, fluorouridine-5-triphosphate is incorporated into RNA replacing uridine triphosphate, producing a fraudulent RNA, and interfering with RNA processing and protein synthesis.[2]

Numerous adverse effects have been reported during 5-FU treatment, including myelosuppression, cardiac toxicity, mucositis, hand–foot syndrome, and diarrhea. The cytotoxic mechanism of 5-FU has been elucidated and is related to modifications in the efficacy of the catabolic pathway of this drug.[3,4]

The key enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD),[5] the initial and rate-limiting enzyme in the 3-step pathway that converts uracil and thymine to β-alanine and β-aminoisobutyrate, respectively. Total DPD deficiency has been reported in pediatric patients presenting with high concentrations of uracil and thymine in urine, blood, and cerebrospinal fluid; this condition can be accompanied by varying levels of neurological abnormalities.[6]

Understanding the mechanism of action and the catabolic pathway of 5-FU has represented a key step for developing new strategies to use this drug. Because 70%–80% of the administered 5-FU is degraded in vivo by DPD, the determination of DPD activity in patients is an index of 5-FU degradation.[7–9] Several studies have documented that cancer patients with a complete or near-complete deficiency of DPD

activity are at risk of developing severe toxicity, including death, after 5-FU administration.[10,11] DPD activity can be detected in a variety of tissues, with the highest levels found in liver and monocytes. To ascertain DPD deficiency, peripheral blood mononuclear cells (PBMCs) or fibroblasts are usually analyzed.[12] These methods include enzymatic assays with radiolabeled thymine or 5-FU,[13] real-time quantitative polymerase chain reaction (PCR) of DPD messenger RNA[14] or gene analysis,[15] and measurement of the dihydrouracil to uracil ratio in plasma.[16]

We describe a novel approach based on the use of intact PBMC to measure the 5-FU degradation rate using a sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method. The aim of the study was to demonstrate that intact PBMC can be an effective tool to study and evaluate the catabolism of 5-FU, reflecting the efficiency of the whole enzymatic machinery involved in the metabolism of the drug. The aim was to show that this procedure could be used to set up a personalized therapy for each patient receiving the drug.

MATERIALS AND METHODS

Reagents and Calibrators

High-performance liquid chromatography (HPLC)–grade methanol and formic acid were purchased from Carlo Erba (Rodano, Italy) and Merck (Darmstadt, Germany), respectively. Phosphate-buffered saline (PBS) was purchased from Eurobio (Les Ulis, France). Separating lymphocyte medium was obtained from GE Healthcare (Uppsala, Sweden). Stock solution of 5-FU (50.0 mg/mL) was provided by TEVA (TEVA srl, Milan, Italy). A 5-FU working solution (100 μg/mL) was prepared by dilution with PBS. 5-Bromouracil (Sigma-Aldrich, St Louis, MO) was used as internal standard (IS) at a concentration of 40 μg/mL in 1% formic acid.

Study Design

The study involved 47 patients, with a histologically confirmed diagnosis of adenocarcinoma of the colon or rectum, from the Medical Oncology Unit of Sant'Andrea Hospital, between January and April 2008. Treatment was given entirely as an outpatient. On day 1 of each 2-week cycle, patients received oxaliplatin 100 mg/m^2 given as an intravenous infusion over 2 hours together with leucovorin 100 mg/m^2 over 2 hours and then 5-FU 400 mg/m^2 as a bolus injection, followed by a 46 hours continuous infusion of 5-FU at a total dose of 2.4 g/m^2. According to Common Terminology Criteria for Adverse Events, 39 patients [mean age 62.2 ± 13.4 years, male to female (M:F) = 19:20] showed only grade 1 toxicity, 5 patients (mean age 63.1 ± 6.5 years, M:F = 2:3) presented a mild grade of toxicity, and 3 patients (mean age 62.5 ± 7.8 years, M:F = 3:0) were affected by high-grade 5-FU toxicity. Mild-grade toxicity was diarrhea of grade 3, dehydration, and vomiting of grade 2. In these patients, chemotherapy dose was reduced by 25%. Severe 5-FU toxicity was acute renal failure requiring hospitalization; mucositis, dehydration, and hyponatremia of grade 3 in excess of 7 days; and grade 3 skin toxicity. This toxicity required a hospital stay >20 days. In all patients with severe toxicities, the chemotherapy administration was discontinued. Blood samples were collected between 9 and 10 AM to avoid changes in DPD activity due to its circadian variation[17] and were processed immediately to isolate PBMC. After 14 days of 5-FU treatment, the rate of 5-FU degradation in intact PBMC was measured.

In patients with mild and high toxic effects, the genomic polymorphisms were investigated, analyzing the splice-site variant IVS14+1G>A in *DPYD* gene that is responsible for the deleterious effect on DPD enzyme activity and is the most common single-nucleotide polymorphism (SNP) detected in patients with 5-FU toxicity.[18] In 1 case, DPD activity was assessed also in another laboratory, using a method validated previously.[19,20] The study received the approval of our local Institutional Review Board and patients gave written informed consent.

Isolation of Human PBMC

PBMCs were isolated from 15 mL of EDTA-anticoagulated blood using Ficoll gradient. After 30 minutes centrifugation at 500*g* (22°C), the PBMC ring was collected, diluted with PBS to a final volume of 50 mL, and centrifuged at 350*g* for 15 minutes at room temperature. The pellet was washed once more with PBS and finally resuspended in ~250 μL of PBS. An aliquot (20 μL) was used for cell counting (Sysmex XE 2100, TOA Medical Electronics, Kobe, Japan), and the volume was then adjusted with PBS to give a concentration of 2–4 × 10^6 cells per 64 μL, to be used for the assay. Samples containing less than 2 × 10^6 cells per 64 μL were discarded (<0.5%).

Determination of the Rate of 5-FU Degradation

The rate of 5-FU degradation was determined by measuring the decrease of a fixed concentration of 5-FU added to the solution of intact PBMC. The reaction mixture was obtained by mixing 64 μL of the cell suspension, containing about 2–4 × 10^6 freshly prepared PBMC, with 16 μL of 50 μg/mL 5-FU (10 μg/mL, final concentration). The reaction was carried out in an Eppendorf tube incubated in a thermomixer at 37°C for 0, 0.5, 1.0, and 2.0 hours; the reaction was stopped by adding 150 μL of 1% formic acid containing IS. Drug stability was assessed by incubating 5-FU in the absence of PBMC.

A calibration curve was determined for each analysis batch. Standard matrix was obtained by vortexing 150 μL of 1% formic acid containing IS with 64 μL of PBMC solution, and then adding 16 μL of 5-FU (from 20 to 0.156 μg/mL, final concentration).

The standards and samples were centrifuged for 5 minutes at 20,000*g* and 20 μL of supernatant was injected into the LC–MS/MS. To calculate the rate of 5-FU degradation, the concentration of the drug after 2.0-hour incubation was subtracted from the calculated concentration at 0 time. Degradation rate was expressed as nanogram per milliliter of 5-FU degraded per minute × 10^6 cells.

Statistical Analysis

Statistical analyses were carried out using the Statistical Package for Social Sciences v.16 (SPSS, Inc, Chicago, IL).

Data were presented as mean ± SD. Method comparisons were performed using the paired *t* test, linear regression, and Bland–Altman plot. Fixed and proportional errors were determined from the 95% confidence limits around the slope and intercept functions of the linear regression and around the mean for Bland–Altman plot. The differences between groups were compared using the Mann–Whitney–Wilcoxon test for non-normally distributed data. Statistical significance was expressed as $P < 0.05$ for a 2-tailed distribution.

HPLC–MS/MS Analysis

The HPLC–MS/MS system consisted of a Q Trap 2000 triple quadrupole/ion trap mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Turbo Spray ion source operated at 400°C. The system included an Agilent 1100 HPLC pump, degasser, autosampler, and column heater. The HPLC separation was performed using a 150 × 2 mm Luna 5 μm C_{18} (2) 100 Å column (Phenomenex, Torrance, CA). Solvent A consisted of 0.05% formic acid and solvent B 100% methanol. Elution was performed at a flow rate of 200 μL/min with the following procedure: 0% solvent B for 4 minutes, 75% solvent B for 2 minutes, then re-equilibration with solvent A for 8 minutes, and the total run time was 16 minutes. Injection volume was 20 μL. Between injections, the autosampler syringe was washed once with methanol. The elution times were 3.71 minutes for 5-FU and 9.16 minutes for IS.

The Q1 and Q3 quadrupoles were tuned for unit mass resolution. The instrument was operated in positive-ion mode. Fragmentation was accomplished with a collision energy of 23.8 eV; nitrogen was the collision gas. Data were acquired and processed with Analyst 1.4.2 software (Build 1162; Applied Biosystems).

HPLC-UV Analysis

For the method comparison, a slight modification of a previously published method[21] was used. In brief, the 39 patients with low toxicity were analyzed by both an in-house HPLC/UV method and the LC–MS/MS method. The HPLC system included a Knauer K-1101 pump, a model 231 Gilson autosampler, and a Knauer UV K-2501 detector with a 140-nL cell. The mobile phases were MilliQ water (solvent A) and acetonitrile/methanol 1:1 (solvent B). Chromatographic separation was performed using a 25 × 3.2 mm, ALLTIMA (Alltech) 5 μm, C_{18} column. Elution was performed at a flow rate of 400 μL/min with the following procedure: 0% solvent B for 10 minutes, 90% solvent B for 5 minutes, and then re-equilibration with solvent A for 10 minutes. Injection volume was 20 μL and total analysis time was 25 minutes between injections. The absorbance was measured at 266 nm. In these conditions, the retention times of 5-FU and IS (5-bromouracil, 40 μg/mL in 1% formic acid) were 9.3 and 14.5 minutes, respectively. Peak integration was performed using Eurochrom HPLC Software-Version 2.05 (Knauer, Berlin, Germany).

Pyrosequencing Analysis

Genomic DNA was isolated from peripheral blood using the X-tractor Gene system (Corbett Life Science, Brisbane, Australia). The splice-site polymorphism IVS14+1G>A in the *DPYD* gene was analyzed using the commercial kit for fluoropyrimidine response (Diatech, Jesi, Italy) according to the manufacturer's protocol. Briefly, the region covering the SNP of interest was amplified by real-time PCR using specific primers, one of which was biotinylated. The amplicon was then sequenced using the Pyrosequencer PyroMark ID system (Biotage AB and Biosystems, Uppsala, Sweden). Single-stranded DNA was isolated from the PCR reaction using the Pyrosequencing Vacuum Prep Workstation (Biotage AB) and Streptavidin Sepharose TM High Performance beads (Amersham Biosciences) that bind to the biotinylated primer. After washing in 70% ethanol, incubation in denaturing buffer, and flushing with wash buffer, the beads were released into a 96-well plate containing annealing buffer and the specific sequencing primer (Diatech). Annealing was performed at 80°C for 2 minutes followed by cooling at room temperature. Then, real-time sequencing was performed.

Analytical Validation

Specific tandem mass spectrometric conditions were optimized using a 10 μg/mL 5-FU solution at a flow rate of 10 μL/min. The method was validated in accordance with the Food and Drug Administration guidelines for bioanalytical method validation and also based on the articles of Shah et al.[22,23] Full validation included linearity, limit of detection (LOD), lower limit of quantification (LLOQ), within-day and between-day precision and accuracy, and stability after sample preparation.

The linearity of calibration curves was established by injecting standard mixtures in matrix at different values. LOD was defined as the lowest detectable amount of 5-FU that gave a signal to noise ratio of 3. The LLOQ was defined as that amount of 5-FU which could be determined with acceptable precision (relative SD, RSD < 20%) and accuracy (percentage of deviation <20%, relative error). Accuracy and precision of the method were assessed from the results of standard matrix, included the LLOQ, and analyzed 3 times in quintuplicate.

The within-day precision (% RSD) and accuracy (% RE) were determined by measuring 4 amounts of 5-FU in 5 batches of triplicates within a day. The within-day precision was calculated for each batch as the % RSD and the overall precision as the grand mean of each % RSD over all 15 results.

The between-day precision and accuracy were calculated by analyzing 4 amounts of 5-FU in triplicates for 5 consecutive days. The between-day precision was calculated as the grand mean of % RSD, derived from the mean of each triplicate per run, over the SD of all the runs.

The accuracy was calculated for each batch or run as the mean value found relative to the theoretical value. The overall accuracy was the grand mean of each % RE to the theoretical value.

RESULTS

Assay Characteristics

The method presented here describes the use of living PBMC to determine the 5-FU degradation rate. Freshly prepared intact PBMCs were incubated with 5-FU (10 μg/mL) and drug concentration was measured at different incubation times (0, 0.5, 1, and 2 hours) by LC–MS/MS. Figure 1 shows

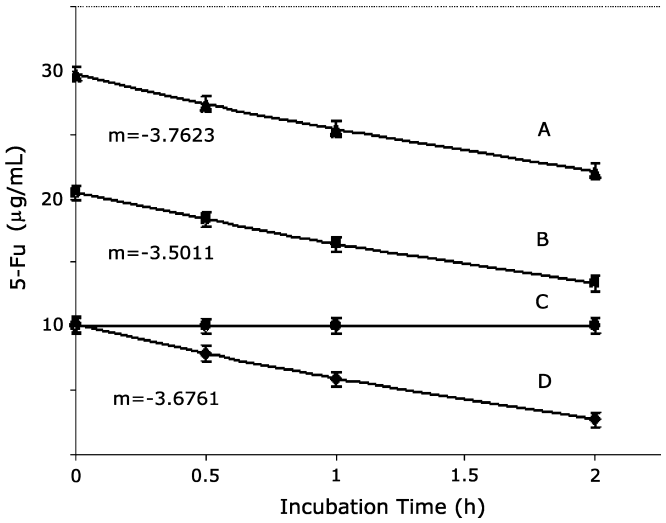

FIGURE 1. 5-FU degradation as a function of incubation time. Different concentrations of 5-FU were incubated in the presence (A, B, and D) or absence (C) of PBMC from 5 patients with no 5-FU toxicity. A, Thirty micrograms per milliliter 5-FU (final concentration); (B) 20 µg/mL; (C and D) 10 µg/mL. SD for all the points was within ±0.6 µg/mL; m, slope.

the degradation rate of 5-FU by PBMC, from 5 patients with low toxicity, using different drug concentrations. When cells were incubated with 10, 20, or 30 µg/mL of 5-FU, drug degradation was linear between 0 and 2 hours. 5-FU was not degraded in the absence of PBMC. Therefore, we decided to use a concentration of 5-FU of 10 µg/mL in our assay, and drug degradation was calculated after 2-hour incubation. The concentration of 10 µg/mL (corresponding to 76.8 µM) is comparable to those (20–25 µM) used in previously described methods[7,10,19] and is about 17 times the K_m value for DPD (3–4 µM).[24,25]

The number of PBMC in the assay (2–4 × 10^6 cells) corresponded to about 150–300 µg protein per sample, similar to the amount (100–300 µg/protein) used in previously described methods.[7,10,19] To exclude the possibility that the apparent 5-FU decrease over time was due to a nonspecific trapping of the drug in the cell membranes, the pellet obtained after lysis and centrifugation was extensively sonicated. No detectable 5-FU was found in the cell debris. The degradation rate determined was similar using an amount of PBMC ranging from 2 to 5 × 10^6 cells (data not shown).

The use of intact PBMC to analyze 5-FU degradation did not require the addition of cofactors because they were present in the cells at physiological concentration. To rule out the possible loss of cofactors during the 2-hour incubation time, 2 parallel assays were run. PBMCs were divided into 2 aliquots. In the first aliquot, 5-FU degradation rate was determined as described. The second aliquot was maintained at 37°C for 2 hours, then 5-FU was added, and its degradation rate was determined after an additional 2 hours. These 2 procedures gave identical results: 1.44 and 1.47 ng/mL of 5-FU degraded per minute × 10^6 cells, respectively.

We considered the possibility that the addition of 1% formic acid to stop the reaction was not sufficient to precipitate all the proteins in the sample, thus affecting the LC–MS/MS analysis. Two parallel assays were run. In 1 assay, the reaction was stopped by adding 1% formic acid and the second using 150 µL of acetonitrile. In the latter case, the supernatant was evaporated in a speed-vac system, resuspended in 200 µL of 0.05% formic acid, and then analyzed. No significant differences were detected using these 2 lytic procedures, so the first, simpler, procedure was used.

In 39 patients with no 5-FU toxicity, the mean 5-FU degradation rate was 1.85 ± 0.50 ng·mL^{-1}·min^{-1} × 10^6 cells and the protein amount was about 0.1 mg. This value corresponds to 142 ± 38 pmol·min^{-1}·mg^{-1} of proteins, which is comparable to the mean value of 197.22 ± 11.34 pmol·min^{-1}·mg^{-1} of protein reported by Di Paolo et al.[19]

In samples from 3 patients characterized by high toxicity after the first chemotherapy cycle, we observed a significant decrease in 5-FU degradation rate values: 0.08, 0.14, and 0.39 ng·mL^{-1}·min^{-1} × 10^6 cells (Fig. 2). Pyrosequencing analysis for the characterization of *DPYD* SNP revealed that 2 of them were heterozygous for the IVS14+1G>A mutation. The sample, in which this *DPYD* mutation was not observed, was analyzed in another laboratory (Department of Internal Medicine, University of Pisa) by a previously published method.[19,20] A severe grade of DPD deficiency was observed.

The 5 patients with mild toxicity showed a 5-FU degradation rate decreased with respect to patients without signs of toxicity and higher than those with high 5-FU toxicity (0.84, 0.95, 0.83, 0.89, and 0.91 ng·mL^{-1}·min^{-1} × 10^6 cells) (Fig. 2). The mutation in the *DPYD* gene was not detected in any of them, using pyrosequencing analysis.

The comparison of the 5-FU degradation rate values between the group of patients with high toxicity and those with low toxicity gave a statistically significant difference, $P < 0.0043$, whereas the patients with mild toxicity versus low toxicity gave a $P < 0.0003$ (Fig. 2).

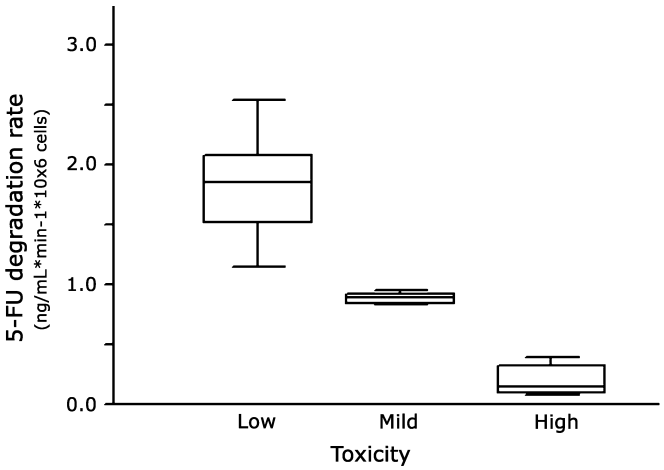

FIGURE 2. Comparison of the 5-FU degradation rate values between patients with low toxicity (n = 39) and those with high (n = 3) and mild (n = 5) 5-FU toxicity. The correlations give a significant difference: high toxicity versus low toxicity, $P < 0.0043$; mild toxicity versus low toxicity, $P < 0.0003$. The Mann–Whitney–Wilcoxon test was used.

LC–MS/MS Parameters

The MS/MS conditions, that is, ion fragments, declustering potential, entrance potential, collision energy, and collision cell exit potential for 5-FU and IS, are shown in Table 1. 5-FU identification relied on monitoring of 2 unique ion fragments. The chromatographic run was conducted in 16 minutes, and the methanol gradient used in the analysis was enough to eliminate interferences from nonpolar compounds or PBS. A representative HPLC chromatogram is shown in Figure 3.

Linearity and Detection Limits

Linear responses were observed in the 5-FU concentration range 0–40 μg/mL. The equation of linear through 0 regression obtained for these value ranges was $y = 157 \times$ ($r = 0.9999$). The accuracy and precision of each point on the standard curve were <15% RE and RSD and were used to calculate the accuracy and precision of the method by their analysis for 3 times in quintuplicate. LLOQ was 0.156 μg/mL with a % RSD of 6.20 and % RE of −1.19. By calculating a signal to noise ratio of 3 (S/N = 3), 5-FU LOD was 0.0625 μg/mL.

Precision and Accuracy

The within-day precision and accuracy were determined by measuring 4 amounts of 5-FU (0.156, 0.195, 0.78, and 1.95 μg/mL) in 5 batches of triplicates within a day. The between-day precision and accuracy were evaluated at the above amounts of 5-FU on 5 consecutive days in triplicate. The results are shown in Table 2.

Ion suppression due to the presence of PBS or other compounds was investigated according to Annesley.[26] PBS was found to cause a decrease of the baseline at 1.5 minutes. By adjusting the chromatographic conditions, the elution times of 5-FU and IS were delayed enough to avoid interference. No significant ion suppression was observed due to endogenous compounds.

Stability of 5-FU After Sample Preparation

The stability of 5-FU at room temperature was investigated by analyzing samples at time 0 and after 24 hours. No significant loss in the signal was detected. Furthermore, the stability was also investigated at 4°C and at −20°C, with no effect on signal detection at 3 days and more than 1 month, respectively.

TABLE 1. Monitored Ion Transitions and Their Parameter Settings

Analyte (Precursor Ion) (*m/z*, amu)	Fragment (*m/z*, amu)	DP (V)	EP (V)	CE (V)	CXP (V)
5-FU (131.00)	113.80	39.00	9.00	23.75	2.07
5-FU (131.00)	58.00	39.00	9.00	40.72	1.75
5-Bromouracil (192.70)	175.90	40.30	7.26	22.75	2.71
5-Bromouracil (192.70)	119.80	40.30	7.26	40.24	2.22
5-Bromouracil (192.70)	149.60	40.30	7.26	29.91	2.34

CE, collision energy; CXP, collision cell exit potential; DP, declustering potential; EP, entrance potential.

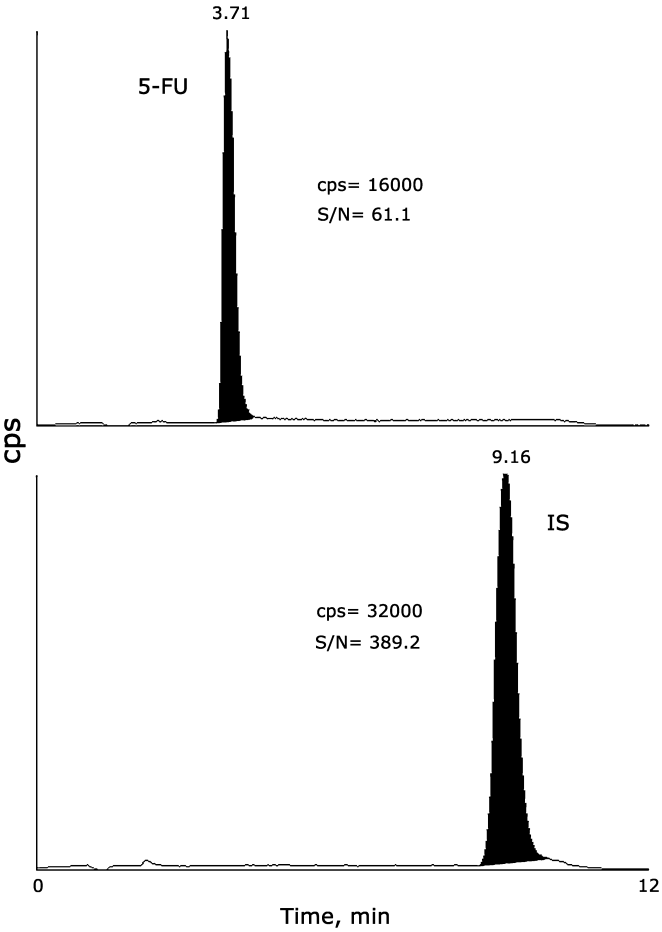

FIGURE 3. Multiple-reaction–monitoring chromatograms of an injected standard solution of 5-FU and 5-bromouracil (IS). HPLC retention times are 3.71 minutes for 5-FU and 9.16 minutes for IS. MS/MS transitions: 5-FU, *m/z* 131.00→113.80; IS, *m/z* 192.70→175.90; cps, count per second; S/N, signal to noise ratio.

Method Comparison

Samples from 39 patients with no 5-FU toxicity were analyzed using both MS/MS and UV detection. In the comparison, the in-house HPLC-UV assay was designated as reference method. Linear regression between the LC–MS/MS and HPLC-UV method for 5-FU gave a slope of 0.9759 (95% confidence interval, 0.9081–1.044; $P < 0.0001$), an intercept of 0.06056 (95% confidence interval, −0.06788 to 0.1890; $P = 0.3483$), and a coefficient of determination (R^2) of 0.9584.

The paired *t* test gave a calculated value for *t* of 0.995 ($P = 0.3259$), showing no significant differences between the 2 methods (critical *t* value for a $P < 0.05$ is 2.01). Furthermore, the Bland–Altman plot showed that no systematic differences existed between the 2 methods and that both methods yielded comparable results (Fig. 4).

DISCUSSION

We describe a method to assess 5-FU degradation rate by intact PBMC. Relevant to this method was the demonstration

TABLE 2. Within-Day and Between-Day Precision and Accuracy

5-FU (μg/mL)	Batch/Day 1 (n = 3) RSD%	1 (n = 3) RE%	2 (n = 3) RSD%	2 (n = 3) RE%	3 (n = 3) RSD%	3 (n = 3) RE%	4 (n = 3) RSD%	4 (n = 3) RE%	5 (n = 3) RSD%	5 (n = 3) RE%	Precision (RSD%) Intraday	Precision (RSD%) Interday	Accuracy (RE%)
0.156	2.5	−1.0	12	5.8	5.0	4.5	7.1	−8.0	7.7	−2.5	8.3	5.6	−0.2
0.195	3.4	6.9	3.9	5.2	7.2	12.5	5.7	0.9	9.2	5.6	6.4	3.9	6.2
0.78	4.1	5.9	5.7	2.4	4.1	10.9	7.4	8.6	4.5	−2.0	6.4	4.9	5.1
1.95	1.0	0.8	1.5	1.7	1.8	2.1	1.2	0.4	1.6	1.0	1.4	0.7	1.2

that intact PBMC freshly prepared from patients can degrade 5-FU added in vitro efficiently. The assay consists of 3 steps: (1) PBMC isolation from peripheral blood, (2) PBMC in vitro incubation with 5-FU for different times, and (3) determination of 5-FU amount to calculate the degradation rate.

A specific feature of this assay is that the rate of 5-FU degradation by intact PBMC is a measure of the whole machinery involved in drug catabolism. This means that the effects of all the degrading enzymes and the different metabolic pathways are considered globally. Furthermore, the use of intact PBMC does not require the addition of DPD cofactors as they are present in the cells at their physiological concentrations. Because we determined the degradation rate of 5-FU and not the enzymatic activity or the rate of product formation, it is not easy to compare our data with those reported in the literature. However, we can calculate that the mean value of 5-FU degradation rate in patients with low toxicity, that is, 1.85 ± 0.50 ng·mL^{-1}·min$^{-1} \times 10^6$ cells (approximately 0.1 mg soluble proteins), corresponds to a degradation rate of 142 ± 38 pmol·min^{-1}·mg^{-1} of protein, which is comparable to the mean value of product formation of 197.22 ± 11.34 pmol·min^{-1}·mg^{-1}, as reported by Di Paolo et al.[19]

The 8 patients with severe or mild 5-FU toxicity showed a significant decrease of 5-FU degradation rate in intact PBMC assay. Genotyping analysis of *DYPD* gene revealed a IVS14+1G>A mutation in only 2 cases. This is not surprising because, although a large number of mutations in *DPYD* gene have been reported,[18] only the IVS14+1G>A mutation is usually analyzed in clinical practice.

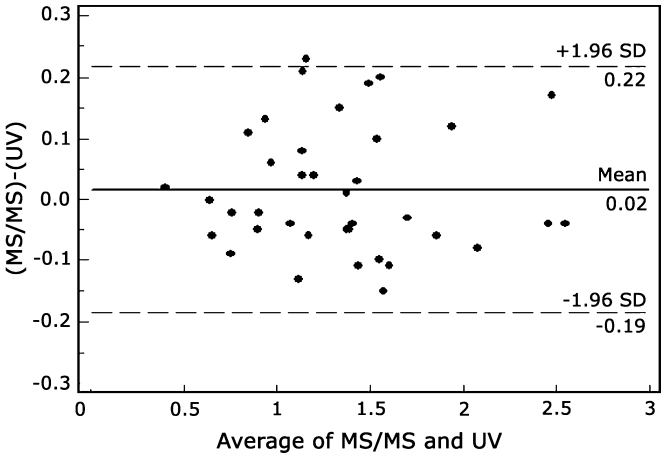

FIGURE 4. Bland–Altman plot in which the 5-FU concentration differences between the 2 methods are plotted against their average.

The comparison of 5-FU degradation rate in intact PBMC between patients with low toxicity and those with high or mild toxicity shows a significant difference, suggesting that the method could allow the detection of those patients with an altered 5-FU metabolism. Furthermore, as shown in Figure 2, a significant difference in the degradation rate is evident between patients displaying high and mild toxicity: This result correlates with the clinical outcome.

To set up a reference range of 5-FU degradation rate in intact PBMC and a threshold to identify patients with an altered 5-FU metabolism, a larger clinical study would be required. The protocol could be performed within a working day, and the results could be delivered in very short time, offering the possibility to adjust 5-FU therapy individually.

In this study, we determined 5-FU amounts by both LC–MS/MS and HPLC-UV with similar results. However, compared with the HPLC-UV assay, the LC–MS/MS method displays a shorter run time and a higher precision and accuracy. Radiochemical assays are highly sensitive, but the use of radiolabeled compounds requires specific instrumentation and safety procedures.[7,13] Furthermore, DPD polymorphism analysis[14,15] gives only an indirect predictive response about the possible DPD deficiency because messenger RNA levels and/or gene polymorphisms do not always correlate with the enzymatic activity.

The MS/MS method described here is highly specific in that it is based on the monitoring of two 5-FU ion fragments. The application of a multiple-reaction–monitoring method allows the analysis of this drug from complex mixtures, such as PBMC extracts, by combination of both HPLC separation and ion fragment identification.

CONCLUSIONS

Personalized medicine, in particular therapeutic drug monitoring, could have an expanding role in clinical practice. Our method allows a rapid determination of 5-FU degradation rate by intact PBMC, as a useful prescreening test for evaluating the possible toxicity of this drug in patients affected by oncological diseases before treatment.

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